THE EFFICACY OF NANOPARTICLES CONTAINING *TALAROMYCES FLAVUS* IN INHIBITING THE GROWTH OF *VERTICILLIUM DAHLIAE*, THE CAUSAL AGENT OF COTTON WILT DISEASE

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ABSTRACT

In Iran, during conducting significant research, the importance of Talaromyces flavus antagonistic fungus has been known to inhibit the growth of some of the important soilborne plant pathogens such as Rhizoctonia solani, Verticillium dahliae and Fusarium oxysporum. Also, according to the results of previous studies, preparation above-mentioned fungal bio formulation was possible with substrates in the form of various plant residues such as rice bran, wheat straw and corn wood using them in the greenhouse and field, in addition to reducing. The occurrence of diseases such as the seedling death, Verticillium and Fusarium wilt in cotton, sugar beet, potato, and greenhouse tomato and cucumber, increased yield also observed. According to the results obtained to this stage of the research, the commercialization of the bio-formulation of this fungus is very importance. Since the marketing is considered as one of the important factors for the continuation of commercialization, consideration of the type of bio formulation with easy applications can greatly affect the attraction of relevant consumers and a successful marketing. Therefore, in light of the recent advances in the application of nanotechnology in various sciences, it seems necessary in the further research to prepare different nano formulations of the biological agent with an emphasis on the ease of use, and study their efficacy in biological control of the plant diseases. In this study, we prepared nano-bio-formulations including two types of nanocapsules (F1 and F3), a type of nano emulsion (F2), and a powder type nano formulation (F4) form T. flavus fungus. Three and six months after the production, laboratory tests of nanoformulations were performed in a completely randomized design in terms of the efficiency in inhibiting the colony growth of Verticillium dahliae. The results showed that nanopowder was more effective than other nanoformulations in term of inhibiting the colony growth of the pathogen.

KEYWORDS: Inhibitory Effect, Nanoparticle, Talaromyces Flavus & Verticillium Dahliae

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INTRODUCTION

During the research conducted in Iran, the desired results of *Talaromyces flavus* antagonistic fungus on controlling some of the most important soilborne plant pathogens such as *Verticillium dahliae*, *Verticillium albo-atrum*, *Fusarium oxysporum* and *Rhizoctonia solani* has been proven in several crops including cotton, sugar beet, potato, and greenhouse tomato and cucumber (Ardekani *et al.*, 2009; Shahraki *et al.*, 2009; Heydari and Pessarakli, 2010; Kakvanet *et al.*, 2013; mansoori *et al.*, 2013).

Also, the application of this fungus in fields as reproduced solid fermentationon plant residues or their mixture with peat soil reduced the incidence of disease and increased yield in the above products; so that in cotton

we had 50% reduction in the percentage of Verticillium wilt, 37% reduction in the seedling death and 30% increase in yield; in potato we had 40% reduction in the disease percentage and 17% increase in yield (Naraghi *et al.*, 2014b); in sugar beet we had 93 % increase in the number of healthy seedlings and 50% increase in yield (Naraghi *et al.*, 2014a); in tomato we had 27% reduction in the disease severity percentage and 23% increase in yield (Farhang Niya *et al.*, 2015) and in greenhouse cucumber we had 30% reduction in disease severity percentage and 7% increase in yield. Since in the mass production and commercialization of biological agents the marketing and attracting the attention of relevant consumers is very important (Husen *et al.*, 2006; Alimi *et al.*, 2009; Kaewchai *et al.*, 2009; Pereira *et al.*, 2009) currently, the commercialization of the biological agent *T. flavus* and producing its various bio formulation, including nanoformulations, seem sessential.

In recent decades, nanotechnology has dramatically advanced in various fields of pharmaceutical chemistry, medicine, and agricultural chemical pesticides. The issue that necessitates research and development in the field of nanopesticides is the pest resistance phenomenon to pesticides; therefore, the introduction of nanopesticides to researchers will boost research and development in this relatively new field. Considering the environmental problems and costs associated with the consumption of large quantities of pesticides, as well as the problems caused by the resistance of pests to these pesticides, research, and development in the field of nanopesticides can be considered as a necessity.

The use of biodegradable polymers in producing nanoemulsions and nanocapsules with high efficiency made of natural and biodegradable materials can be an effective step in this field. In order to increase the efficiency and reduce the environmental hazards, the encapsulation formulation seems the best option (Maji *et al.*, 2014). Therefore, the bioformulation production in the form of nano and micro provides a controlled ability, increased strength and stability, and protects the active ingredient under adverse environmental conditions such as light and moisture. Also, the use of nano-encapsulated formulations can help reduce pesticides' dosage and costs, protect the environment and reduce its environmental hazards, as well as better product exports (Martin *et al.*, 2010).

Nanoparticles have a larger surface-volume ratio than micro-particles, which increases their active surface and controlled release. Also, the other advantage of a nanoparticle is that this does not stimulate the human and animal body and rapidly exits the body (Guan *et al.*, 2008).

Nanocapsule technology that contains nanoscaled fungicide or pesticide molecules is one of the methods for producing pesticides' formulation that removes more easily and quickly pests (Guan *et al.*, 2008). An emulsion is a heterogeneous system composed of two non-mixing liquids, one of which is dispersed in droplets in another. Nanoemulsion is an emulsion with a droplet size of about nanometers and typically range from 20 to 200 nm (Ostertag *et al.*, 2012). The unique structure and characteristics of nanoemulsions have produced many advantages in many industries compared with conventional emulsions. The applications of nanoemulsions in industries can be their role in encapsulating and controlling the release of super compounds, such as essential oils, vitamins, and etc. (Hofman and Kan, 2014).

Recently, we witnessed the tendency to produce nanoparticles that are more biodegradable and have high efficiency. Therefore, the use of biodegradable polymers in the production of high-efficiency nanoemulsions and nanocapsules made of natural and biodegradable materials such as essential oil of medicinal herbs can be an effective step in this field. The use of nano pesticides, the production, application and environmental considerations indicate that research and development can be effective in reducing pest resistance phenomena to pesticides. The nano formulations prepared by microorganisms, such as bacteria and fungi, are used in the biological struggle against insects and facilitate the entry of

agents to the insect's body (Salukhe et al., 2011). The nano formulation of the insecticide Pirimphos makes the formulation be kept well in the dark and more stable (Wan et al., 2010).

The nanoformulation of the essential oil extracted from plants against pests resistant to conventional pesticides was more than five times more potent than pesticides (Rajakumara and Rahuman, 2011). The nano formulation of Permethrin by evaporation of oil solvent in water could have a better effect on controlling the larvae of the mosquito Culex (Anjali et al., 2010). The degradation properties of Bacillus thuringiensis have been investigated using nanoparticles of chitosan polymer on the larvae of the mosquito Anopheles (Zhang et al., 2015). Also, using dextrose and gelatin polymers bio-pesticides containing Beauveria, Metharhisium and Paecilomyces were prepared against Hypothenemus hampei (Niranjana et al., 2004).

Also, Beauveria bassiana formulations containing silver nanoparticles were used as anti-larvae (Prabakaran et al., 2016). In a study on disinfectant effects of barley and sunflower seeds with silver nanoparticles containing fungicide on mycorrhizal symbiosis, it was found that in treating seeds by nanofungicide in comparison to the conventional fungicide, the amount of minerals' absorption by the root and consequently vegetative traits have been significantly increased. Covering materials for encapsulation include gum, starch, gelatin and polymers. Recently, chitosan and phospholipid are also used. According to recent research, the use of the nanoencapsulation technique and the components in storage pests' management can play an important role in increasing the efficiency and durability (Prasad et al., 2014).

A large number of the plant compounds with insecticidal properties are highly evaporated and susceptible to decomposition. The loading of plant compounds into nanoparticles causes releasing under control, delaying decomposition and evaporation. There are few papers about the effect of nanoemulsion insecticides and nanocapsules loaded by compounds with insecticidal properties against pest insects (Margulis-Goshen and Magdassi, 2013).

There are several methods for producing nanoparticles or nanocapsules. Among them, there is polymerization method, which is one of the quickest methods, and continuous aqueous phase is mixed with continuous organic phase (Kirthi et al., 2011). Also, the methods of coacervation and ion gel formation using hydrophilic biodegradable polymers such as chitosan, gelatin, sodium alginate, which is in fact a mixture of two aqueous phases that is placed in a polymeric phase such as chitosan and sodium aljinate, and in this method a positive charge in a polymer is mixed with a negative charge of crosslinks, such as sodium triphosphate or calcium chloride, and forms capsules (Ebrahimnejad et al., 2011).

So far, there have been two reports in the field of medicine and agriculture for the preparation of nano fungicides of the studied fungus genus (Penicillium) (Priyadarshini et al. 2014; Khan and Jameel, 2016). In the field of medicine, for the preparation of nano fungicides against the pathogen fungus Candida albicans, the fungus Penicillium fellutanum has been used. In the field of agriculture, for the preparation of nano fungicides against some plant pathogens, Talaromyces flavus has been used.

In the last 20 years, there have been significant reports on the preparation of bioformulations containing antagonistic fungi using solid and liquid fermentation and their optimization at various stages of production (Pascual et al., 1999; Budge and Whipps, 2001; Schuster and Schmoll, 2010; Caramenz et al., 2012; Kakvan et al., 2013; Sargin et al., 2013). Pascual et al. (1999), for example, succeeded in producing solid bio-formulations from Epicoccum nigrum fungus on wheat, and after analyzing alcoholic solutions containing glycerol, mannitol and arbitol on sporulation of this fungus, the most significant increase in sporulation was reported in glycerol. Also, Sargin et al. (2013) attempted to increase the

efficiency of the bio-formulation containing *Trichoderma harzianum* EGE-K38, compared with the different methods of drying this bio-formulation. The study results have also shown that the application of compounds containing minerals such as manganese, iron, zinc and phosphorus in the production of biological formulations containing antagonistic fungi has increased their stability (Vasane and Kothari, 2008; Lee and Lee, 2009).

So far, overseas bio-formulations such as Ketomium containing *Chaetomium globosum* and *Ch. Cupreum*; Promote containing *T. harzianum* and *T. Virus*; Soil Gard containing *Gliocladium virens*; Trichodex containing *T. harzianum*, *Pisolithus tinctorius* and *Glomus intraradices*; Trichodermin containing *T. harzianum* and Protus WG containing *Talaromyces flavus* have been commercially registered (Merwel et al., 1974; Koch, 1999; Kaewchai et al., 2009). In Iran, literature review to make bio-formulations containing various *T. flavus* isolates is as follows:

T. flavus antagonistic fungus in Iran for the first time was isolated from the soil around the roots of cotton located in a field at Golestan Province's Karkandeh Research Station. The results of laboratory tests to investigate the effects of inhibitory mechanisms of different isolates of *T. flavus* on the growth of some pathogens in several crops showed that among the studied mechanisms, including mycoparasitism and the production of volatile and non-volatile compounds, the mechanism of production of non-volatile compounds caused the highest percentage of cotton *Verticillium* wilt pathogen growth inhibitory (*V. dahliae*). In another study, it was found that common antagonistic mechanisms among *T. flavus* isolate associated with three crops of greenhouse potato, tomato and cucumber were mycoparasitism and producing volatile compounds for *V. albo-atrum*, mycoparasitism for *F. oxysporum*, and producing non-volatile compounds for *R. solani*.

In other studies, to use the mentioned fungus in the greenhouse and field, the bio-formulation containing it was prepared using solid substrates including bred fertilizer by worms, wheat straw, wheat bran, rice bran, wheat straw combined with wheat bran, perlite mixed with sugar supplement and peat soil mixed with sugar supplement. Meanwhile, the best substrate was peat soil mixed with sugar supplement in terms of the efficiency in increasing sporulation and stability of *T. flavus* isolates related to cotton, potato, and rice bran, and greenhouse tomato and cucumber for *T. flavus* isolates.

The application of rice bran substrate for *T. flavus* reproduction has been reported in the field of biological control of garlic white rot and reinforce its vegetative traits through the use of this fungus. Also, for biological control of the seedling death and root rot in cotton and cucumber, this substrate has been used for the reproduction of antagonistic bacteria such as *Pseudomonas fluorescens* (Ardekani et al., 2010; Khabbaz and Abbasi, 2014; Mehdizadehnaraghi et al. 2015).

The results of greenhouse experiments in the field of biological control the greenhouse potato, tomato and cucumber *Verticillium* wilt caused by *V. albo-atrum* by bio-formulations containing different isolates of *T. flavus* showed that these isolates reduced the disease index and increased vegetative traits such as the root length, crown length, height, fresh weight and dry weight of the above plants significantly (Naraghi et al., 2010a, 2010b and 2010c; Naraghi et al., 2012a and 2012b).

On the other hand, according to the results of laboratory and greenhouse studies in the field of controlling the pathogen of fusarium wilt of the greenhouse tomato and cucumber by bio-formulations containing different isolates of *T. flavus*, in addition to determining the most effective bio-formulation, the method of using each of them was also found in tomato fields and cucumber greenhouses.

Also, the field studies were conducted on the possibility of the biological control of cotton verticillium wilt and seedling death; sugar beet seedling death; potato Verticillium wilt and Fusarium wilt of tomato and greenhouse cucumber using *T. flavus* bio-formulation; and the results showed that the use of this bio-formulation, in addition to the significant reduction of the disease index, has also led to a significant increase in yield (Naraghi et al., 2014a and 2014b; Farhang Niya et al., 2015).

MATERIALS AND METHODS

Producing Nanocapsule Formulation Containing *Talaromyces Flavus*

The production of nanocapsules is a combination of the polymerization and networking methods by making changes that are consistent with the conditions of the growth of the biological fungus (changes in the amount or type of polymer, surfactants and oils, fatty acid, mixer round and temperature). In the polymerization process, the organic phase was made up of vegetable oil with a mixture of biological fungus added in an aqueous phase consisting of hydrophilic polymer monomers such as a mixture of one of two polymers of poly urea formaldehyde or alginate, starch and chitosan. Then, cross linkers such as calcium chloride, as well as surfactants and the accompanying materials, and fatty acid oils were added to both phases, and the homogenization was performed at a temperature of 35 °C with a homogenizer with 5,000 to 10,000 rpm. Finally, the network polymer particles formed in the form of capsules around the particles of the biological fungus.

Producing Nanoemulsion Formulation Containing *Talaromyces Flavus*

The Saelf-assemblage pattern was used to prepare a nanoemulsion formulation containing *T. flavus* biological fungus. The final formulation was a nano emulsion formulated in a bio-compatible formulation of plant oil hydrophobic nanoparticles. The compositions of this formulation is as follows: the active ingredient of the biological fungus and hydrophobic plant oil, such as castor, tween surfactant, carboxymethyl cellulose viscosity material, coconut moisturizer, di ethanol amide fatty acid, stabilizers such as polyvinyl alcohol and linker such as calcium chloride and biocompatible polymers such as ethylene glycol and starch. First, a uniform solution of biocompatible polymers was prepared, then surfactants such as twin and the accompanying materials were added to the solution and a completely homogeneous mixture of polymer and solvent was prepared with a homogenizer with 2000 to 12000 rpm at 25 °C. Then, suspensions containing spores of the biological fungus plus fatty acids' oil of castor and coconut were added as drops. At the next stage, a crosslinker (calcium chloride) was added to both phases to form the nanoparticles around the spores of the biological fungus. Finally, the nanoparticles around spores of the biological fungus were covered.

Producing Nanopowder Formulation Containing *Talaromyces Flavus*

In nanopowder formulation, a suspension containing the biological fungus spores was prepared in the aqueous phase, including Maltodextrine, Xanthan Gum, Fatty Acid, Ethanol Amide and Oleic Acid, and completely powdered after placing in a homogenizer with 2000 to 12000 rpm at 25 °C.

Compare the Efficiency of Different Nanoformulations of *T. Flavus* in Terms of Inhibiting the Growth Colony of *Verticillium Dahliae*

Study the efficiency of *T. flavus* various nanoformulations in term of inhibiting the colony growth of *Verticillium dahliae* (the causal agent of Cotton Verticillium Wilt disease), will be done in one year and a half. This comparison began three months after the production, and will continue for up to fifteen months at intervals of three months. To examine each

nanoformulations, a petri dish containing PDA culture medium with an assumptive line was divided into half, in a half we put a 0.5 millimeter piece of the pathogen by Cork Borer, and in the other half, we put 0.1 gram of a nanoformulation. For each of the aforementioned pathogens, a separate study was conducted in a completely randomized design with five treatments (four new nanoformulations and control) and three replications. For the control petri dish, we put the pathogen piece only in half, of the petri dish, and in the other half, we did not put the determined amount of nanoformulation. In order to determine the inhibitory percentage of each studied pathogen, seven days after the presence of the pathogen and formulation on the petri dish, the colony diameter of the pathogen was measured in the treatment and control. According to the following formula, the inhibitory percentage of the pathogen colon growth was calculated by nanoformulations.

The inhibitory percentage= $\frac{\text{the pathogen colony diameter in control} - \text{The pathogen colony diameter in the treatment containing nanoformulation}}{\text{The pathogen colony diameter in control}} \times 100$

Data analysis and comparison of the means of growth inhibitory percentage of the pathogen colon was performed by nano formulations with Duncan's multiple range test using software MS TAT C. Up to this stage of the study, the efficiency of various nanoformulations has been studied in terms of inhibiting the growth of some plants' pathogens up to six months after the production.

In this study, we have used *Verticillium dahliae* in the collection of the useful microorganism research laboratory at the Iranian Research Institute of Plant Protection which the pathogenicity has already been proven.

Also, it should be noted that in this study, it was not possible to determine the efficacy of the previous bio-formulation in inhibiting the pathogens' growth. The main substrate of the previous bio-formulation of *T. flavus* are rice bran, which, upon placing on the petri dish, bran particles containing the fungus are spread on the petri dish and quickly develops over the entire surface of the petri dish without any pathogens' growth, so it is possible to measure the pathogen colon diameter and calculate the pathogen inhibitory percentage by this formulation. There was not. Therefore, we will compare the efficiency of different nanoformulations with the bio-formulation based on previous technical knowledge on controlling some important plant diseases under the greenhouse conditions.

RESULTS AND DISCUSSIONS

Compare the Efficacy of Different nanoformulations of *T. Flavus* in Terms of Inhibiting *Verticillium Dahliae* Colony Growth

In the first and second three months after the production, the study of the efficiency of nanoformulations in inhibiting the colony growth of *V. dahliae* (Figures 1 and 2) showed that the inhibitory effect reduced from the first three months to the second three months in nanocapsule 1, nanoemulsion and nanopowder, while it increased in nanocapsule 2 (Table 1). The efficacy of different nanoformulations in inhibiting the colony growth of *V. dahliae* in the first and second three months after the production was significant at 1% probability level.

In the first three months after the production, the comparison of the inhibitory effect means of *V. dahliae* colon growth from each formulation showed that the formulations were in three statistical groups and the most effective one in terms of inhibitory effect was nanopowder (Table 1). In the first three months, microsclerotia melanization was also observed in nanocapsule 1, and nanoemulsion and nanocapsule 2 (Fig. 2). However, in the second three months after the production, microsclerotia melanization was not observed in any of the nano formulations (Fig. 3). In the second three months, the comparison of the means of inhibitory levels of *V. dahliae* from each formulation also showed that

formulations were in three statistical groups and the most effective nanoformulation in term of the efficiency in inhibiting the colony growth of *V. dahliae* was nanopowder (Table 1).

Figure 1: Observation of the Inhibitory Effect of *Verticillium Dahliae* Colony Growth by Different Nano Formulations on PDA Culture Medium in the First Three Months After Production: Control, F1 (Nano Capsule Treatment 1), F2 (Nano Emulsion Treatment), F3 (Nano Capsule Treatment 2) and F4 (Nano Powder Treatment)

Figure 2: Observation of the Inhibitory Effect of *Verticillium Dahliae* Colony Growth by Different Nanoformulations on PDA Culture Medium in the Second Three Months after Production: Control, F1 (Nanocapsule 1), F2 (Nanoemulsion), F3 (Nanocapsule 2) and F4 (Nanopowder)

Table 1: Comparison of *Verticillium Dahliae* Colony Growth Inhibitory in Treatments Containing Various Nanoformulations

Formulation	The Mean <i>Verticillium Dahliae</i> Colony Growth Inhibitory (%)	
	The First Three Months	The Second Three Months
F1(nano capsule 1)	26.93c	42.85a
F2(nano emulsion)	25.24c	26.28c
F3 (nano capsule 2)	44.68b	25.00c
F4 (nano powder)	52.39a	60.00a

* No statistically significant difference is among the means of the same statistical letters at 1% probability level.

Figure 3: Formation of Growth Inhibitory Areas in the Petri Dish Containing the Cultural Medium of *Candida Albicans* using the Pills Coated with Nanoformulation Containing *Penicillium Fallutanum* (Khan and Jameel, 2016)

CONCLUSIONS

The results obtained from the inhibitory effect of nano formulations prepared on the growth of some plant pathogens in the present study were consistent with the results of previous studies (Khan and Jameel, 2016) on the inhibitory effect of nano formulations containing *Penicillium lowerutanum* fungus of nanoformulation on *Candida albicans* fungus, as in vitro in Petri dishes, the zones associated with the lack of growth of pathogenic fungus is shown around nano formulation pills in this study (Figure 3).

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